IN THE UNITED STATES PATENT AND TRADEMARK OFFICE		
	Application Number	09/258,217
	Filing Date	26 February 1999
	First Named Inventor	Mark T. KEATING
	Group Art Unit	1633
	Examiner Name	S. CHEN
	Attorney Docket Number	2323-127
Title of the Invention:	**MICE WHICH ARE +/- OR -/- FOR THE ELASTIN GENE AS MODELS FOR VASCULAR DISEASE**	

DECLARATION UNDER 37 C.F.R. 1.131

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

We, Mark T. Keating and Dean Y. Li, the applicants for the above-identified patent application, declare as follows:

1. That sometime prior to May 1998, we conceived of and reduced to practice the invention described therein, i.e., (a) mice which were either ELN +/- or ELN -/- with a phenotype having an increased number of elastic lamellae and having arterial occlusion and (b) the central role that elastin plays in the pathogenesis of obstructive arterial disease.

2. The preparation of these mice were all performed by us in the laboratories of the University of Utah in Salt Lake City, Utah.

3. The results of the experiments reducing the invention to practice were described in a manuscript entitled "Elastin is an Essential Determinant of Arterial Morphogenesis: Implications for Vascular Disease" submitted to the University of Utah's Technology Transfer Office prior to May 1998. This manuscript was coauthored by us as well as eight noninventors. A copy of this manuscript is attached as Exhibit A. This manuscript was published in *Nature* on May 21, 1998 (Li et al. (1998), "A Elastin is an Essential Determinant of Arterial Morphogenesis," *Nature* 393 276-280).

4. It is further declared that the accompanying exhibit is not a complete record of the Applicants' data concerning the invention of the above-mentioned patent application and are not necessarily meant to represent the earliest date of conception. The accompanying exhibits are

presented solely to prove conception prior to the date of prior art (Morris) cited by the Patent Examiner in the Office Action dated 10 July 2001.

The Declarants further state that the above statements were made with the knowledge that willful false statements and the like are punishable by fine and/or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statement may jeopardize the validity of this application or any patent resulting therefrom.

Dated: 8-28-02 _____

Mark T. Keating

Dated: 3/1/2002 _____

Dean Y. Li

original

U-2511

ELASTIN IS AN ESSENTIAL DETERMINANT OF ARTERIAL MORPHOGENESIS: IMPLICATIONS FOR VASCULAR DISEASE

Dean Y. Li[1,3], Gilles Faury[2*], Benjamin Brooke[3*], Elaine C. Davis[4*], Douglas G. Taylor[3], Beth B. Boak[5], Peter Stenzel[6], Ernst Eichwald[7], Robert P. Mecham[2], and Mark T. Keating[1,3,5]

[1]Cardiology Division, University of Utah Health Sciences Center,
[3]Department of Human Genetics, Eccles Institute of Human Genetics,
[5]Howard Hughes Medical Institute, Salt Lake City, Utah 84112-0533

[2]Department of Cell Biology and Physiology, and Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

[4]Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9039

[6]Department of Pathology, Oregon Health Sciences University, Portland, Oregon 97201-309

[7]Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, Utah, 84112

*Contributed equally

EXHIBIT
A

HEADING: Elastin, the main component of arterial extracellular matrix, is thought to have a purely structural role. Consistent with this view, elastin hemizygous mice maintain arterial extensibility by increasing the number of elastic lamellae during development. However, mice lacking elastin die of obstructive arterial pathology. This pathology results from subendothelial proliferation and reorganization of smooth muscle, cellular changes similar to those observed in atherosclerosis. Thus, elastin is a molecular determinant of arterial morphogenesis and likely plays a central role in vascular disease.

Vasculogenesis begins early in vertebrate development and culminates in the formation of a complex network of arteries, veins, and capillaries. Once formed, the gross and microscopic structure of this network is stable unless disrupted by disease. Genetic and cell culture studies have begun to identify molecular determinants of vasculogenesis, and these determinants have defined three distinct stages of vascular development[1,2]. In the first stage, splanchnic mesoderm coalesces to form simple tubes of endothelial cells. Vascular endothelial growth factor defines this stage[3,4,5]. The second stage involves the recruitment of mesenchymal cells by the endothelium, a process coordinated by angiopoietin and platelet-derived growth factor[6-9]. In the third stage, mesenchyme differentiates into smooth muscle and extracellular matrix deposition begins. Transforming growth factor beta has been implicated in this stage[2,10]. After the third stage of vascular development, arterial smooth muscle cells exit the cell cycle and vascular structure is stabilized[11-13]. The molecular determinants of this final stage are unknown.

There is growing evidence that the extracellular matrix regulates cellular function during organogenesis. Fibronectin, vitronectin, collagen, and other extracellular matrix proteins bind to integrins on the surface of cells [14,15], providing morphogenic signals that regulate cell proliferation, migration, and differentiation[16,17]. Disruption of fibronectin in mice causes dramatic developmental abnormalities, including failure to develop a notochord and somites[18]. Null mutations in genes encoding fibronectin receptors, or integrins, lead to embryonic or perinatal death from developmental abnormalities resembling those observed in mice lacking fibronectin[19,20]. Not all cell-matrix interactions, however, are necessary

for normal morphogenesis. For example, disruption of vitronectin, tenascin C, and integrin alpha 1 have no apparent affect on development[21-23].

Elastin is the dominant arterial extracellular matrix protein[24]. This protein is encoded by a single gene, and organized into polymers that form concentric rings of elastic lamellae around the arterial lumen. Each elastic lamella alternates with a ring of smooth muscle, forming a lamellar unit. The function of elastic fibers was thought to be purely structural, providing tensile strength and resiliency to the aorta and other arteries. Because of its structural role, investigators believed that disruption of elastin would lead to dissection of arteries. This view was supported by studies associating decreased elastin content and increased elastase activity with arterial aneurysms in humans and other species[25,26]. In addition, disruption of collagen I and fibrillin, prominent arterial extracellular matrix proteins, resulted in rupture of blood vessels in mice and humans[27,28]. Our human molecular genetic studies demonstrated, however, that *ELN* mutations do not cause arterial dilatation, but instead cause an obstructive arterial disease, supravalvular aortic stenosis (SVAS)[29,30]. To define the role of *ELN* in arterial development and disease, we generated mice hemizygous (*ELN* +/-) and homozygous (*ELN* -/-) for an *ELN* null mutation. From characterization of these mice, we conclude that elastin has two distinct functions. One is to provide arterial elasticity and ameliorate wall stress. A second is to control smooth muscle proliferation and organization during late arterial development. Elastin, therefore, is a molecular determinant of arterial morphogenesis, defining a fourth stage of development, the stage of arterial stabilization.

RESULTS

Targeted disruption of murine *ELN*

Murine *ELN* genomic clones were isolated from a SV/129 λ FIX II library using an *ELN* cDNA clone. A targeting vector was constructed to delete 4.0 kb of the promoter and exon 1, resulting in a null mutation (Fig. 1a). The vector was electroporated into R1 embryonic stem cells and homologous recombinants were isolated by positive-negative selection[31]. Three out of 160 clones were identified as homologous recombinants by Southern analysis. These three clones were microinjected into C57BL/6 blastocysts and implanted in pseudopregnant females[32]. All three independent cell lines generated chimeras that transmitted the null *ELN* allele. The resulting *ELN* +/- mice were mated to generate *ELN* -/- mice (Fig. 1b).

The disrupted *ELN* allele is a null as demonstrated by the absence of *ELN* mRNA and protein in *ELN* -/- mice (Fig. 1c-h). Elastin expression during fetal development is largely confined to the vascular system and begins in the last third of gestation[24]. *In situ* hybridization demonstrated the absence of *ELN* mRNA in the aorta of *ELN* -/- mice and its presence in *ELN* +/+ and *ELN* +/- mice (Fig. 1c, d and data not shown). Northern analysis showed an absence of *ELN* mRNA in *ELN* -/- mice at birth, (P0.5: Fig. 1e, f). Hart staining demonstrated the absence of elastin protein in *ELN* -/- mice (Fig. 1g, h).

By contrast with *ELN* +/+ mice, Northern analysis of *ELN* +/- mice revealed a 47% decrease in *ELN* mRNA (Fig. 1e, f). To determine if the structure of elastic lamellae in *ELN* +/- mice was affected, we examined aortic cross sections by electron microscopy (Fig. 2). Elastic lamellae in *ELN* +/- mice (Fig. 2a, d) were approximately 50% thinner than those in *ELN* +/+ mice (Fig 2b, e). These data indicate that elastic lamellae in *ELN* +/- mice are structurally abnormal, presumably due to reduced synthesis of elastin mRNA and protein during development.

Changes in structure and function of *ELN* +/- aortas

ELN +/- mice were identical to *ELN* +/+ mice in gross appearance, behavior, and life expectancy (Table 1). To determine the affect of *ELN* hemizygosity on arterial structure, we examined the vascular system using light and electron microscopy. The ascending and descending aorta were examined at 5-14 months. It is generally believed that the number of lamellar units in an artery is fixed, species-specific and genetically determined[33,34]. We discovered, however, that aortas dissected from *ELN* +/- mice had additional lamellar units. Consistent with previous work [34,35], we found that *ELN* +/+ aortas had 5.4 $\pm$ 0.5 and 8.4 $\pm$ 0.5 layers of elastic lamellae, respectively, in the descending and ascending aorta (Fig. 3 and Table 2). By contrast, cross sections of *ELN* +/- mice revealed an increase in the number of lamellar units to 7.3 $\pm$ 0.6 and 10.5 $\pm$ 0.5 layers respectively ($p<0.005$). This represented an increase of 35% and 25% for the descending and ascending aorta of *ELN* +/- mice. There were no histological differences identified in the cardiac, pulmonary, gastrointestinal, renal, endocrine, musculoskeletal, or integumentary

systems. Our results indicate that *ELN* +/- mice develop aortas with an increased number of elastic lamellae.

To determine the physiological consequences of structural changes observed in *ELN* +/- mice, we measured aortic diameter and extensibility at varying intraluminal pressures (Fig. 4). At a physiological pressure of 100 mm Hg, diameter and extensibility of *ELN* +/+ and *ELN* +/- mice were similar. At 125 mm Hg and above, however, the pressure-diameter curves diverged with a marked reduction in extensibility of *ELN* +/- aortas ($p<0.05$). These data indicate that *ELN* +/- mice maintain aortic diameter and extensibility at physiological pressure.

ELN +/- mice predict SVAS pathology

SVAS is a human obstructive arterial disease that results from *ELN* hemizygosity[30]. To determine if developmental changes observed in *ELN* +/- mice were also present in SVAS, we examined aortic segments from affected individuals (n=2 for affected, n=3 for controls: Fig. 5 and data not shown). We studied regions of the aorta that were free of discrete stenosis. The number of lamellar units in controls was consistent with previous reports[36]. By contrast, the aortic wall of individuals with SVAS was thicker and contained 2.5 fold more lamellar units (152 ± 27.6 vs 62 ± 8.7:$p<0.025$). These data indicate that humans with *ELN* hemizygosity, like their murine counterpart, develop an increased number of aortic elastic lamellae.

Arterial occlusion in *ELN* -/- mice

ELN -/- mice survived gestation but died by P4.5 (Table 1). To determine the consequences of elastin deficiency, we performed a developmental survey (Fig. 6a-h). The histological appearance of ascending aortas from *ELN* -/- and *ELN* +/+ mice were indistinguishable through embryonic day 17.5 (E17.5). Beginning at E17.5, the outer and inner aortic diameter of the *ELN* -/- mice became progressively smaller. In *ELN* +/+ mice, by contrast, the aorta continued to expand. The diameter of the arterial wall in *ELN* -/- mice became progressively thicker after E17.5. This change was caused by the subendothelial accumulation of cells, a process that eventually obliterated the vascular lumen. Cell counts of the ascending aorta demonstrated a significant difference between *ELN* +/+ and *ELN* -/- mice at E17.5 (Fig. 6i). By P2.5 there were 76% more cells in the *ELN* -/- aortas. Increased cellularity was also observed in other vessels, including the descending thoracic aorta, pulmonary, brachiocephalic and carotid arteries (data not shown). These data indicate that late arterial development is disrupted in mice lacking elastin.

The accumulating cells in aortic cross sections of *ELN* -/- mice were morphologically distinct from smooth muscle in normal arteries. To determine the identity of these cells, we immunostained aortic sections with alpha smooth muscle actin antisera. The accumulating subendothelial cells present in *ELN* -/- aortas were positively stained (Fig. 7a, b). Sagittal sections demonstrated that these cells were longitudinally oriented (Fig 7c, d). By contrast, smooth muscle cells in the media of control animals were circumferentially oriented. Thus, luminal obliteration in

ELN -/- aortas results from subendothelial accumulation and reorientation of smooth muscle.

To examine the mechanism underlying smooth muscle accumulation in *ELN* -/- mice, we stained aortic sections with antisera to proliferating cell nuclear antigen (PCNA; Fig. 7e, f). At E17.5, the number of cells staining for PCNA was greater in *ELN* -/- mice than in *ELN* +/+ mice (88% versus 35%). There was a gradient of staining, with subendothelial cells of *ELN* -/- aortas staining prominently. Intestinal sections were used as controls, confirming that PCNA was present in the crypts and absent in the villi (data not shown). These experiments indicate that subendothelial proliferation of smooth muscle is the mechanism underlying the arterial pathology observed in *ELN* -/- mice.

Arterial obliteration without inflammation

The hallmark of obstructive arterial disease is subendothelial accumulation of smooth muscle cells[37,38]. It is generally believed that recurrent endothelial injury, thrombosis, and inflammation induce this pathology[37]. To determine if these processes were responsible for smooth muscle accumulation in *ELN* -/- aortas, we used histochemistry, immunohistochemistry, and electron microscopy to examine aortic sections. Sagittal and cross sections of aortas stained with alpha smooth muscle actin antisera revealed a single uninterrupted layer of unstained cells, the endothelium. The identity of these cells were further defined using von Willebrand factor antisera (Fig. 7g, h). No evidence of endothelial damage or disruption was observed. Microscopic examination

of *ELN* -/- aortas stained with hematoxylin and eosin showed no evidence of inflammation (Fig 6). Gross and histological examination of arteries revealed no evidence of thrombosis. Because collagen is a ligand for integrin receptors and can modulate proliferation of smooth muscle, we looked for increased collagen using Masson trichrome[39]. No increase was found (Fig. 7i, j). Electron micrographs confirmed the integrity of the endothelium, the lack of inflammation and normal distribution of other extracellular matrix proteins, including collagen (Fig. 2c, f). Thus, endothelial damage, thrombosis, inflammation, and fibrosis are not responsible for subendothelial accumulation of smooth muscle in *ELN* -/- mice and, therefore, are not necessary for inducing these pathological changes. Instead, *ELN* disruption is sufficient for initiating subendothelial accumulation and reorganization of smooth muscle.

DISCUSSION

Physiological regulation of arterial development

It was previously thought that the number of lamellar units in an arterial wall was fixed, species-specific and genetically determined[33,34]. Our data are not consistent with this view. We found that *ELN* +/- mice develop arteries with a marked increase in the number of lamellar units. Examination of arterial specimens obtained from individuals with SVAS, a human disorder caused by *ELN* hemizygosity, also revealed a dramatic increase in the number of elastic lamellae. Thus, the number of lamellar units in an arterial wall is not fixed or species-specific, but is modulated during development by elastin.

The mechanism underlying elastin's affect on lamellar development is unknown, but likely involves elastin content and wall stress. We found that the increased number of lamellar units observed in *ELN* +/- mice was associated with reduced *ELN* mRNA and thinning of each elastic lamella. It is likely that a quantitative reduction in elastin during arterial development resulted in lamellae with reduced extensibility. These changes would lead to increased arterial wall stress, which is determined by arterial pressure and diameter and inversely proportional to the number of lamellar units and the tensile strength of each unit[40]. However, the extensibility and diameter of *ELN* +/- arteries were normal at physiological pressures. Thus, mice with abnormal elastic fibers maintain arterial extensibility by increasing the number of lamellar units during development. The alternative explanation, that elastin specifies the number of lamellar units by regulating gene expression during arterial

development, is unlikely given the extracellular location of elastin. Our model is also supported by physiological studies showing that the relationship between wall stress and the number of elastic lamellae in an artery is remarkably constant despite enormous variation in arterial diameter and stress across species[34]. We conclude that the number of lamellar units in an arterial wall is modulated during development by the quantity of elastin and the physiological force of wall stress.

Our work defines a novel pathology in a human obstructive arterial disease, supravalvular aortic stenosis[41,42]. Because of arterial pathology in *ELN* +/- mice, we reexamined apparently unaffected aortic sections from individuals with SVAS. We discovered a dramatic increase in the number of elastic lamellae. Thus, humans also respond to reduced elastin content by increasing the number of lamellar units during development. The change in humans (150% increase) was more dramatic than mice (25-35% increase), reflecting large differences in wall stress between these species (110,000 versus 7,800 dynes/cm). We could not examine extensibility in humans with SVAS, but progressive arterial pathology in affected individuals suggests that compensatory changes are inadequate, leading to discrete stenoses.

Elastin defines a fourth stage of vasculogenesis

Our studies of *ELN* -/- mice define a novel stage of vasculogenesis. In this fourth and final stage, the extracellular matrix matures, smooth muscle cells exit the cell cycle and become organized, and the artery stabilizes[1,2,11,12]. Arterial development in mice lacking elastin was

indistinguishable from controls until E17.5, indicating that elastin has little or no affect on the first three stages of vasculogenesis. During subsequent development, however,*ELN* -/- aortas became smaller and thicker, arterial diameter declined and the lumen eventually obliterated. The cellular mechanism underlying these changes was subendothelial accumulation of arterial smooth muscle, a process that involved cell proliferation, reorganization and reorientation. Thus, elastin is a molecular determinant of late arterial morphogenesis, stabilizing arterial structure by controlling proliferation and organization of smooth muscle.

By contrast with the mechanism of pathology in *ELN* +/- mice, it is unlikely that wall stress has an important role in arterial obliteration in *ELN* -/- mice. As noted above, wall stress is directly proportional to arterial pressure and luminal diameter and inversely proportional to wall thickness. As wall thickness increases and luminal diameter decreases, wall stress declines and approaches zero. Thus wall stress cannot account for luminal obliteration in *ELN* -/- mice.

The molecular mechanism through which disruption of elastin leads to proliferation and reorganization of arterial smooth muscle is unknown. One likely mechanism is that elastin provides morphogenic signals during arterial development by binding to receptors on the surface of cells. Direct evidence supporting this hypothesis includes ultrastructural data showing specific contact points between smooth muscle cells and elastic lamella, cell biological data demonstrating the ability of elastin peptides to modulate cellular function, and biochemical data identifying an elastin receptor[43-45]. Precedence for this model is provided by the integrin family of receptors

that regulate cellular proliferation, migration, differentiation, and organization by binding to fibronectin and other extracellular matrix proteins[16,17]. A second possible mechanism is that elastin acts by providing a structural cage, directly preventing smooth muscle from proliferating and migrating.

Elastin disruption and vascular disease

Obstructive arterial diseases, like atherosclerosis and restenosis, are the main cause of morbidity and mortality in industrialized nations[46]. Although much is known about vascular risk factors like hypercholesterolemia, hypertension, diabetes, and cigarette abuse, the pathogenic mechanism through which these diverse factors converge to give a common pathology is unknown[38,47]. The hallmark of obstructive arterial disease is subendothelial accumulation of smooth muscle cells, pathology that causes luminal narrowing and life-threatening sequelae[38]. Previous models have focused on endothelial injury, thrombosis and inflammation, processes that are thought to induce cellular accumulation through a complex interplay of cytokines and growth factors[37]. By contrast with these models, we found no evidence of endothelial damage, thrombosis or inflammation in *ELN* -/- mice. Instead, we discovered that disruption of *ELN* is sufficient for inducing smooth muscle proliferation and reorganization. Although disruption of extracellular matrix is a prominent feature of atherosclerosis, this process was not thought central

to pathogenesis[48,49]. We propose that endothelial injury, thrombosis, inflammation, and other vascular risk factors induce subendothelial pathology by disrupting elastin, and that elastin is central to the pathogenesis of obstructive arterial disease.

Methods

Construction of Targeting Vector

Two phage from a λ FIX II library (Stratagene: La Jolla, CA) encompassing exon 1 and spanning a total of 26 kb were isolated, mapped and subcloned. To construct a targeting vector, we used a 6.5 kb *Xba*I fragment as the 5' region of homology and a 4.2 kb *Bam*HI-*Hin*DIII fragment as the 3' region of homology. Positive selection was provided by a 3.1 kb *Sac*I-*Cla*I fragment containing the neomycin resistance gene under the control of the Pol II promoter. These fragments were cloned into the vector TK1-TK2A which provided two thymidine kinase genes for negative selection.

Generation of Mice

Culture, selection of embryonic stem cells, and screening of targeted clones were carried out as previously described[27,28]. Clones were screened by Southern blot analysis of *Xba*I digested genomic DNA probed with a 3.0 kb *Hin*DIII - *Xba*I fragment. Random integration events were excluded using a probe derived from the 3' region of homology. The homologous recombinant clones were injected into blastocysts of C57Bl/6J and transferred into uteri of pseudopregnant C57Bl/6J females. The resulting chimeric animals were backcrossed to C57Bl/6J, and heterozygous mutants were identified by genomic Southern blotting of tail DNA. Heterozygous mice were mated for three generations with C57Bl/6J mice and brother-sister matings were carried out to generate homozygous mutants.

Northern Analysis

Poly A(+) RNA was extracted from the visceral organs of the thorax from P0.5 mice using a Micro-FastTrack Kit (Invitrogen: Carlsbad, CA) . RNA was electrophoresed on a 1.0% denaturing agarose gel, transferred to Hybond filter (Amersham: Arlington Heights, IL), and hybridized with a ^{32}P -labeled 0.85 kb fragment of mouse *ELN* cDNA. Filters were rehybridized with a 1.5 kb fragment of human cardiac actin cDNA

Histological Examination

Mice were fixed overnight in either 4% paraformaldehyde or methyl Carnoys at 4°C and embedded in paraffin. Sections were stained with hematoxylin and eosin, Hart, and Masson-trichrome. Lamellar units and cell numbers were counted on two separate occasions by individuals blinded to the genotype. The statistical significance was calculated by comparison of the means using t test analysis.

Immunohistochemistry

Tissue samples were immunostained with a monoclonal antibody against smooth muscle alpha-actin (clone 1A4, 1:400, Sigma Chemical: St. Louis, MO), human von Willebrand factor antibody (1:1000, DAKO: Carpinteria, CA), or proliferating cell nuclear antigen (PCNA) monoclonal antibody (clone PC10, 3μg/mL, Calbiochem: San Diego, CA).

Biotinylated donkey anti-rabbit antibody (RPN 1004, 1:2000, Amersham) was used as a secondary antibody for von Willebrand factor, and biotinylated goat anti-mouse IgG_{2a} antibody (RPN 1181, 1:100, Amersham) was used as a secondary antibody for smooth muscle alpha-actin and PCNA. Sections immunostained for smooth muscle alpha-actin or von Willebrand factor were developed with 3-amino-9-ethylcarbazole chromagen (DAKO) and counterstained with Mayers hematoxylin (Sigma Chemical). Sections immunostained for PCNA were developed in 3,3'-diaminobenzidine chromagen (Vector Laboratories: Burlingame: CA) and counterstained with methyl green.

Vascular extensibility

The ascending aorta from 5-6 month old mice was cannulated and mounted on the pressure myograph[50]. The vessel was transilluminated under an inverted microscope connected to a CCD camera, allowing the continuous recording of the outer diameter of the vessel. Intravascular pressure was increased from 75 to 175 mm Hg by steps of 25 mm Hg, and arterial diameter was recorded. Extensibility was calculated with using the following formula using 100 mm Hg as an example; extensibility = [(diameter at 125 mm Hg - diameter at 75 mm Hg)/(diameter at 75 mm Hg)] X 100. Statistical analysis was assessed by a four way ANOVA followed by Least Significance Difference test for post ANOVA paired comparisons.

Electron Microscopy

Ascending thoracic aortas were dissected from mouse pups at birth following cardiac perfusion with 3% glutaraldehyde. Aortic segments were sequentially stained with osmium tetroxide, tannic acid and uranyl acetate, then dehydrated and embedded in Epon[35]. Thin sections (60nm) were counterstained with uranyl acetate and lead citrate and examined on a Jeol 1200 electron microscope.

Acknowledgments:

We thank D. Atkinson and H. Katchuk for help with manuscript preparation; M. Parmacek, S. Odelberg, L. Urness, K. Thomas, R. White, S. Prescott, J. Kaplan, and M. Sanguinetti for critical comments; K. Thomas for technical advice. ES cells and chimeric mice were generated in the laboratory of Dr. M.R. Capecchi, University of Utah School of Medicine. This work was supported by the National Institutes of Health, Bristol-Myers Squibb Foundation, and Fondation pour la Recherche Medicale.

Correspondence and requests for materials to Dean Li
dean@howard.genetics.utah.edu

References

1. Hanahan, D. Signaling vascular morphogenesis and maintenance. *Science* **277,** 48-50 (1997).

2. Folkman, J. & D'amore, P.D., Blood vessel formation: what is its molecular basis? *Cell* **87,**1153-1155 (1996).

3. Shalaby, F., Rossant, J., Yanaguchi, T.P., Gertenstein, M., Wu, X-F, Breitman, M.L. & Schuh, A.C. Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. *Nature* **376 ,** 62-66 (1995).

4. Fong, G.-H., Rossant, J., Gertsenstein, M. & Breitman, M.L. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376,** 66-69 (1995).

5. Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertenstein, M., Fahrig, M.,Vandenhoeck, A., Harpal, K., Eberhardt, C., Declerq, C, Pawling, J., Moons, L., Collen, D., Risau, W. & Nagy, A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435-439 (1996).

6. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, S., Powell-Braxton, L., Hillan, K.J. & Moore, M.W. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380,** 439-442 (1996).

7. Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S. Sato, T.N. & Yancopoulos, G.D. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87,** 1161-1170 (1996).

8. Lindahl, P, Johansson, B.R., Leveen, P. & Betsholz, C. Pericyte loss and microaneurysm formation in PDGF-B deficient mice. *Science* **277,** 242-245 (1997).

9. Sato, T.N., Tozawa, Y., Deutsch, U., Wolberg-Buchholz, Fugiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W. & Qin, Y. Distinct role of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376,** 70-74 (1995).

10. Beck, L. & D'Amore, P.A. Vascular development: cellular and molecular regulation. *FASEB J.* **11,** 365-373 (1997).

11. Schwartz, S.M., Heimark, R.L. & Majesky, M.W. Developmental mechanism underlying pathology of arteries. *Physiol. Rev.* **70,** 1177-1209 (1990).

12. Owens, G.K. Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75,** 487-517 (1995).

13. Glukova, M.A., Frid, M.G. & Koteliansky, V.E. Phenotypic changes of human aortic smooth muscle cells during development and in the adult vessel. *Am. J. Physiol.* **261,** 78-80 (1991).

14. Gumbiner, B.M. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84,** 345-357 (1996).

15. Hynes, R.O. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69,** 11-25 (1992).

16. Adams, J.C. & Watt, F.M. Regulation of development and differentiation by the extracellular matrix. *Development* **117,** 1183-1198 (1993).

17. Hynes, R.O. Genetic analyses of cell-matrix interaction in development. *Curr. Opin. Genet. Dev.* **4,** 569-74 (1994).

18. George, E.L., Georges-Labousse, E.N., Patel-King, R.S., Rayburn, H. & Hynes, R.O. Defects in mesoderm, neural tube, and vascular development in mouse embryos lacking fibronectin. *Development* **119,** 1079-1091 (1993).

19. Yang, J.T., Rayburn, H. & Hynes, R.O. Embryonic mesodermal defects in alpha 5 integrin deficient mice. *Development* **119,** 1093-1105 (1993).

20. Yang, J.T., Rayburn, H. & Hynes, R.O. Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development* **121,** 549-60 (1995).

21. Zheng, X., Saunders, T.L., Camper, S.A., Samuelson, L.C. & Ginsburg, D. Vitronectin is not essential for normal mammalian development and fertility *Proc. Natl. Acad. Sci. USA* **92,** 12426-30 (1995).

22. Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T. & Aizawa, S. Mice develop normally without tenascin. *Genes Dev.* **6,** 1821-1831 (1992).

23. Gardner, H., Kreidberg, J., Koteliansky & Jaenisch, R. Deletion of integrin alpha 1 by homologus recombination permits normal murine development but gives rise to a specific deficit in cell adhesion. *Dev. Biol.* **175,** 301-13 (1996).

24. Parks, W.C., Pierce, R.A., Lee, K.A. & Mecham, R.P. Elastin in Advances in Molecular and Cell Biology. *JAI press* **6,** 133-182 (1993).

25. Thompson, R.W. Basic science of abdominal aortic aneurysms: emerging therapeutic strategies for an unresolved clinical problem. *Curr. Opin. Cardiol.* **11,** 504-518 (1996).

26. Terpin, T. & Roach, M.R. A biophysical and histological analysis of factors that lead to aortic rupture in normal and lathyritic turkeys. *Can. J. Physiol. Pharmacol.* **65,** 395-400 (1987).

27. Lohler, J., Timpl, R. & Jaenisch, R. Embryonic lethal mutation in mouse collagen I gene causes rupture of blood vessels and is associated with erythropoietic and mesenchymal cell death. *Cell* **38,** 597-607 (1984).

28. Dietz, H.C. & Pyeritz, R.E. Mutations in the human gene for fibrillin-1 (FBN1) in the Marfan syndrome and related disorders. *Hum. Mol. Genet.* **4,** 1799-1809 (1995).

29. Curran, M., Atkinson, D., Ewart, A., Morris, C., Leppert, M. & Keating, M. The elastin gene is disrupted by a translocation associated with supravalvular aortic stenosis. *Cell* **73,** 159-168 (1993).

30. Ewart, A., Morris, C., Atkinson, D., Jin, W., Sternes, K., Spallone, P., Stock, A., Leppert, M. & Keating, M. Hemizygosity at the elastin locus in a developmental disorder. *Nat. Genet.* **5,** 11-16 (1993).

31. Mansour, S.L., Thomas, K.R. & Capecchi, M.R. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336,** 348-352 (1988).

32. Thomas, K.R. & Capecchi, M.R. Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346,** 847-850 (1990).

33. Katoh, Y. & Periasamy, M. Growth and differentiation of smooth muscle cells during vascular development. *TCM.* **6,** 100-106 (1996).

34. Wolinsky, H. & Glagov, S. A lamellar unit of aortic medial structure and function in mammals. *Circ. Res.* **20,** 99-111 (1966).

35. Davis, E.C. Elastic lamina growth in the developing mouse aorta. *J. Histochem. and Cytochem.* **43,** 1115-1123 (1995).

36. Machii, M. & Becker, A.E. Morphologic features of the normal aortic arch in neonates, infants, and children pertinent to growth. *Ann. Thorac. Surg.* **64,** 511-515 (1997).

37. Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809 (1993).

38. Schwartz, S.M., deBlois, D. & O'Brien, E.R.M. The intima: soil for atherosclerosis and restenosis *Circ.* **77,** 445-465 (1995).

39. Koyama, H., Raines, E.W., Bornfedt, K.E., Roberts, J.M. & Ross, R. Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of cdk2 inhibitor. *Cell* **87,** 1069-1078 (1996).

40. Milnor, W.R. in Principles of Hemodynamic in Cardiovascular Physiology, Oxford University Press, 184-186, 1990.

41. Perou, M. Congenital supravalvular aortic stenosis. *Arch. Path.* **71,** 113-126 (1961).

42. O' Connor, W. Supravalvular aortic stenosis: clinical and pathologic observations in six patients. *Arch. Pathol. Lab. Med.* **109,** 179-185 (1985).

43. Davis, E.C. Smooth muscle cell to elastic lamina connections in developing mouse aorta: role in aortic medial organization. *Lab. Invest.* **68,** 89-99 (1993).

44. Senior, R.M., Griffin, G.L., Mecham, R.P., Wrenn, D.S., Prasad, K.U. & Urry, D. W. Val-gly-val-ala-pro-gly, a repeating peptide in elastin, is chemotactic for fibroblasts and monocytes. *J. Cell Biol.* **99,** 870-874 (1984).

45. Mecham, RP & Hinek, A. Non-integrin laminin receptors in The Laminins (P. ELblom and R. Timpl, eds) Harwood Academic Publishers 1996.

46. "Heart and Stroke Facts: 1996 Statistical Supplement" (American Heart Association, Dallas, TX, 1996).

47. Gibbons, G.H. & Dzau, V.J. Molecular therapies for vascular diseases. *Science* **272,** 689-693 (1996).

48. Glagov, S., Weisenberg, E., Zarins, C.K., Stankunavicius, R. & Lolettis, G.J. Compensatory enlargement of human atherosclerotic coronary arteries. *N. Engl. J. Med.* **316,** 1371-1375 (1987).

49. Galis, Z.S., Sukhova, G.K., Lark, M.W. & Libby, P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atheroscelerotic plaques. *J. Clin. Invest.* **94,** 2493-2503 (1994).

50. Boyle, W.A. & Maher,G.M. Endothelium-independent vasoconstricting and vasodilating action of halothane on rat mesenteric resistance blood vessels. *Anesthesiology* **82,** 221-235 (1995).

Figure Legends

Figure 1. Disruption of murine *ELN*. **a,** Schematic of targeting vector designed to delete *ELN* promoter and exon 1. **b,** Southern analysis of tail DNA digested with *Xba*I from *ELN* +/+, *ELN* +/-, and *ELN* -/- mice . The 9 kb wild type and 6 kb targeted *Xba*I fragments were identified using a probe outside the 3' region of homology. **c, d,** Dark field photographs of *in situ* hybridization from cross-section of embryonic day 17.5 (E17.5) mice using rat elastin cDNA riboprobe labeled with ^{33}P-dUTP. Elastin mRNA is seen in the vasculature of *ELN* +/+ but not *ELN* -/- mice. **e, f** Northern analysis of mRNA from *ELN* +/+, *ELN* +/- ,and *ELN* -/- mice at birth (P0.5) for *ELN* and cardiac actin expression. There was a 47% decrease in *ELN* mRNA in *ELN* +/- mice by phosphorimage analysis. No *ELN* mRNA was detected in *ELN* -/- mice. **g, h,** Hart stain detected elastic fibers (stained dark brown) in ascending aortas from *ELN* +/+ mice at P0.5. No elastic fibers were seen in *ELN* -/- mice.

Figure 2. Electron micrographs of the subendothelial region (**a-c**) and media (**d-f**) of ascending aortic cross sections from *ELN* +/+, *ELN* +/- and *ELN* -/- mice at P0.5. In *ELN* +/+ mice, elastic lamellae were well developed and evenly distributed from lumen to adventitia with circumferentially oriented smooth muscle cells interposed between lamella. Note that elastic lamella were thinner in *ELN* +/- mice and absent in *ELN* -/- mice. Bar = 3.0 μm.

Figure 3. Hart stain of *ELN* +/+ and *ELN* +/- aortas. Ascending (**a, b**) and descending aortas (**c, d**) are shown. Note the increased number of elastic lamellae in *ELN* +/- mice.

Figure 4. Extensibility of *ELN*+/+ and *ELN* +/- aortas. **a,** Pressure-diameter curve. **b,** Extensibility at varying pressures. Note that diameter and extendibility of *ELN* +/+ and *ELN* +/- aortas were similar at physiological pressure, 100 mm Hg. Asterisk indicates $p<0.05$.

Figure 5. Elastin van Gieson stain of descending aorta cross sections from a human with SVAS and a control. Descending aortas were examined 1.0 cm distal to the left subclavian artery. Note marked increase in the number of elastic lamellae in SVAS, a disorder caused by *ELN* hemizygosity.

Figure 6. Developmental comparison of aortic histology in *ELN* +/+ and *ELN* -/- mice. **a-h,** Cross sections of ascending aortas (at the level of the pulmonary artery) stained with hematoxylin and eosin were examined at E15.5, E17.5 , P0.5 , and P2.5. No difference was noted at E15.5. Subsequent timepoints revealed subendothelial accumulation of cells in *ELN* -/- mice (arrows) and obliteration of the lumen by P2.5. **i,** Graphical representation of the increase in cell number in the ascending aorta of *ELN* +/+ and *ELN* -/- mice. The cell numbers begin to differ at E17.5, with $p<0.025$ (*). At P0.5 and P2.5, the differences were significant at $p<0.005$ (**).

Figure 7. Mechanism of luminal obliteration in *ELN* -/- mice. **a, b,** Sections of P0.5 mice immunostained with alpha smooth muscle actin antisera. Note that subendothelial cells stained positively (arrow), indicating that they are smooth muscle. **c, d,** Sagittal sections of ascending aorta at P2.5 stained with hematoxylin and eosin. Note that the subendothelial cells in *ELN* -/- mice were longitudinally oriented (arrow), whereas normal orientation is circumferential. **e, f,** Aortic sections of E17.5 mice were immunostained with PCNA antisera (dark brown nuclei). The percentage of cells stained with PCNA was greater in *ELN* -/- mice (88% versus 35%), indicating increased cell proliferation in *ELN* -/- aortas. **g, h,** Sections of P0.5 mice were immunostained with von Willebrand factor antisera (red). No evidence of endothelial damage was observed. **i, j,** Sections of P0.5 mice incubated with Masson trichrome, which stains collagen green. No collagen deposition was observed.

Table 1: Life expectancy of *ELN* +/+, *ELN* +/-, and *ELN* -/- mice

	+/+	+/-	-/-
Embryonic (E15-5-18.5)	97 (28%)	163 (48%)	82 (24%)
Postnatal			
P0.5	94 (29%)	152 (46%)	81 (25%)
P1.5	19 (24%)	45 (57%)	15 (19%)
P2.5	33 (26%)	74 (59%)	19 (15%)
P3.5	10 (48%)	10 (48%)	1 (4%)
>P3.5	65 (31%)	140 (69%)	0 (0%)

Litters of mice were sacrificed at each timepoint and genotypes were tabulated. Pecentages are calculated in the parenthesis. No *ELN* -/- mice were alive after P3.5.

Table 2: Number of lamellar units in *ELN* +/+ and *ELN* +/- aortas

Mouse ID	Genotype	Age	Ascending	Descending
BL6	WT	5 mo	9	6
390-3	WT	12 mo	8	5
390-10	WT	14 mo	8	6
390-11	WT	14 mo	8	5
1002-7	WT	4 mo	9	6
1029-8	WT	5 mo	8	5
1045-3	WT	5 mo	9	5
		Average:	8.4 ± 0.5	5.4 ± 0.5

Mouse ID	Genotype	Age	Ascending	Descending
390-6	HET	12 mo	11	7
390-8	HET	14 mo	11	7
390-9	HET	14 mo	11	7
705-7	HET	7 mo	11	8
720-2	HET	7 mo	10	8
720-6	HET	7 mo	10	6
1029-4	HET	6 mo	11	8
1029-5	HET	6 mo	10	7
1029-6	HET	5 mo	10	7
1029-7	HET	5 mo	10	7
1045-1	HET	6 mo	11	7
1045-4	HET	6 mo	10	8
		Average:	10.5 ± 0.5	7.3 ± 0.6

Differences in number of lamellar units between *ELN* +/- mice and *ELN* +/+ mice were statistically significant with a $p<0.005$.